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(54) Title: USE OF CYP1B1 INHIBITORS FOR TREATING CANCER

(57) Abstract: CYP1B1 proteins and their role in metabolising or inactivating anti-cancer drugs is disclosed, together with compositions for treating cancer comprising a substance capable of inhibiting CYP1B1 protein and an anti-cancer drug (e.g. docetaxel, paclitaxel, flutamide, tamoxifen, mitoxantrone, doxorubicin or daunomycin).

USE OF CYPIBI INHIBITORS FOR TREATING CANCER

Field of the Invention

The present invention relates to CYP1B1 proteins and
their role in cancer, and more particularly to the use of
inhibitors of CYP1B1 proteins to ameliorate the
inactivation of anti-cancer drugs by CYP1B1 present in
cancer cells.

Background of the Invention

The cytochromes P450 are a multi-gene family of
constitutive and inducible enzymes which have a central
role in the oxidative metabolic activation and
detoxification of a wide range of xenobiotics and several
groups of endogenous compounds active in cell regulation
and cell signalling, including arachidonic acid, steroid
hormones and fatty acids. The major families of P450
involved in xenobiotic metabolism each consist of several
individual forms with different regulatory mechanisms and
substrate specificities. The majority of P450s are
primarily expressed in liver, although individual P450
forms are also expressed in specific extra-hepatic
tissues including small intestine, kidney and lung.

The human CYP1 gene family, which is one of the major
P450 families involved in the metabolism of xenobiotics,
is now known to consist of three individual forms
classified into two sub-families. The CYP1A subfamily
contains two highly homologous and well characterised but
distinct members, CYP1A1 and CYP1A2. CYP1A1 is an
inducible P450 expressed primarily in extrahepatic
tissues while CYP1A2 is a major form of P450 that is
constitutively expressed in liver. There is also a
second human CYP1 subfamily which contains one member,
CYP1B1. This P450 is dioxin-inducible and sequence
analysis of CYP1B1 shows 40% homology with both CYP1A1

and CYP1A2. Although CYP1B1 is assigned to the CYP1 family on the basis of its sequence, it is structurally and functionally distinct from both CYP1A1 and CYP1A2.

5   WO97/12246 (University of Aberdeen) discloses that CYP1B1 is present in a range of tumour cells and proposes the use of this enzyme as a marker for the diagnosis of cancer. This application further discloses therapies for the treatment of cancer based on the presence of CYP1B1

10   in tumour cells. In one embodiment, the application proposes a selective therapy employing drugs which are activated by CYP1B1 present in tumour cells, converting a prodrug into a cytotoxic form capable of killing tumour cells. In other embodiments, WO97/12246 proposes the use

15   of CYP1B1 present in tumour cells as a marker to guide a therapeutic compound selectively to the cells, e.g. by conjugating a drug to a moiety which is capable of specifically recognising the CYP1B1. WO97/12246 further suggests that CYP1B1 may be involved in providing an

20   essential function for tumour cells in inactivating endogenous anti-tumour compounds such as 2-methoxyestradiol. In view of this, the application proposes reducing endogenous CYP1B1 levels in tumour cells, e.g. by using antisense RNA or suicide inhibitors

25   to inhibit CYP1B1 production.

WO00/56773 (University of Aberdeen) relates to the fragments of CYP1B1 for use in raising antibodies capable of specifically binding CYP1B1, and the use of such

30   antibodies for the diagnosis or treatment of cancers linked to enhanced CYP1B1 expression.

Summary of the Invention

Broadly, the present invention is based on the finding

35   that the presence of CYP1B1 in tumour cells contributes

to the resistance of tumour cells to anti-cancer drugs. This opens up the possibility of enhancing the effectiveness of cancer therapies employing these anti-cancer drugs by inhibiting CYP1B1 or reducing its level in tumour cells thereby ameliorating the effect of the CYP1B1 in inactivating or metabolising the drugs.

CYP1B1 is a member of the cytochrome P450 superfamily of enzymes. There have been some reports in the prior art that a number of the other P450 family members are involved in a biotransformation of anti-cancer drugs used to treat a variety of cancers. Cytochrome P450 mediated metabolism generally leads to inactivation or reduced activity of the drug, whereas cyclophosphamide, an inactive pro-drug, must firstly undergo a 4-hydroxylation reaction by cytochrome P450 enzymes before becoming cytotoxic. However, as set out above, there is not a close relationship between CYP1B1 and other P450 family members in terms of their structure or the substrates on which the enzymes act. Accordingly, the finding that CYP1B1 causes drug resistance is not predictable based on the results obtained with other P450 enzymes.

Ovarian cancer causes the greatest number of deaths from gynaecological malignant disease in the developed world. The lack of symptoms in the early stages of ovarian cancer means that up to 80% of newly diagnosed patients will have disease that is advanced, where complete surgical resection is not possible and with an overall five year survival of only 30%. Early stage ovarian carcinoma has a good prognosis with 80% five year survival and generally chemotherapy is not recommended, whereas adjuvant chemotherapy with a platinum-based regimen is advocated for patients with advanced disease. The results disclosed herein confirm that CYP1B1

expression in ovarian cancer shows a strong correlation with patient survival for treatment with paclitaxel. The recent introduction of the taxane, paclitaxel, for the treatment of ovarian cancer may result in a better

5   progression-free and overall survival. Docetaxel, a semi-synthetic taxane, is currently being evaluated for use in the treatment of ovarian cancer. Some of the results disclosed herein examine the ability of CYP1B1 to alter the cytotoxic profile of fourteen anti-cancer drugs

10 commonly used as first line treatment in solid tumours.

In the studies disclosed herein, a Chinese hamster ovary cell line expressing human cytochrome P450 CYP1B1 was used to evaluate the cytotoxic profile of several anti-

15 cancer drugs (docetaxel, paclitaxel, cyclophosphamide, doxorubicin, 5-fluorouracil (5FU), cisplatin and carboplatin) commonly used clinically in the treatment of cancer. The MTT (3-[4,5-dimethylthiazol-2yl]-2,5-diphenyltetrazolium bromide) assay, was used to determine

20 the levels of cytotoxicity. The key finding of this study was that on exposure to docetaxel and paclitaxel, a decrease in sensitivity towards the cytotoxic effects of the drug was observed in the cell line expressing CYP1B1 compared to the parental cell line. This effect was

25 particularly marked for docetaxel (p = 0.03), while the lesser result obtained with paclitaxel may be due to the particular cell line used in the assay or because the metabolic product of paclitaxel retained some cytotoxicity to the cells. Further, this difference in

30 cytotoxicity was reversed by co-incubation of the cells with both docetaxel and the cytochrome P450 CYP1 inhibitor α-naphthoflavone (ANF). This study is the first to indicate, that the presence of CYP1B1 in cells decreases their sensitivity to the cytotoxic effects of a

35 specific anti-cancer drug. Further studies with kidney

tumours showed that addition of the CYP1B1 inhibitor α-naphthoflavone inhibited metabolism of EROD by CYP1B1 in the tumour cells.

5   Several cytochrome P450 enzymes are involved in the metabolism of a range of anti-cancer drugs, such as cyclophosphamide, paclitaxel and docetaxel [9-14]. Cytochrome P450 mediated metabolism usually results in reduced activity or inactivation of the anti-cancer drugs
10   but in some cases bioactivation to a more cytotoxic metabolite occurs. One example of detoxification of anti-cancer drugs can be shown by the taxanes. The major pathway of metabolism of paclitaxel, an anti-cancer drug used in the treatment of breast, ovarian and non-small
15   cell lung cancer, is catalysis by CYP2C8 and involves the hydroxylation of position 6 on the taxane ring [15]. The metabolite 6-hydroxytaxol is 30 fold less cytotoxic than the parent compound paclitaxel [16], and 6-hydroxytaxol is further metabolised by CYP3A4 [13]. Docetaxel, a
20   semi-synthetic taxane, currently undergoing phase II and phase III trials for use in first-line therapy of ovarian cancer is metabolised by CYP3A to apparently less cytotoxic metabolites [12,13].

25   Accordingly, in a first aspect, the present invention provides the use of a substance capable of inhibiting CYP1B1 protein and an anti-cancer drug for the preparation of a medicament for the treatment of cancer.

30   Preferably, the substance capable of inhibiting CYP1B1 protein inhibits the activity of CYP1B1 enzyme in metabolising the anti-cancer drug or a pro-drug form thereof, or a metabolic product of the anti-cancer drug or pro-drug, thereby inactivating, detoxifying or
35   otherwise modifying it so that it loses some or all ·of

its anti-cancer activity (e.g. cytotoxicity to cancer cells), or in the case of a pro-drug, the capacity to be converted to active drug. Examples of CYP1B1 inhibitors are well known in the art and include flavones and flavonoids such as α-naphthoflavone (ANF), accacatin, diosmetin, hesperetin and homoeriodictyol, and 2-ethynylpyrene. See, for example, Doostdar et al, Toxicology, 144:31-38, 2000 and Shimada et al, Chem. Res. Toxicol., 11:1048-1056, 1998. Preferred inhibitors are selective, that is they inhibit CYP1B1 while having a reduced inhibitory effect, or more preferably substantially no effect, on the function of endogenous enzymes present in normal cells. In addition to using known inhibitors, the skilled person can readily screen libraries of compounds to look for further inhibitors for use in accordance with the present invention.

The anti-cancer compound may be a drug or a pro-drug and is preferably a substrate of CYP1B1. Examples of suitable anti-cancer drugs for use in conjunction with the CYP1B1 inhibitors include docetaxel, paclitaxel, flutamide, tamoxifen, mitoxantrone, doxorubicin or daunomycin, and more especially taxanes such as docetaxel and paclitaxel. The results disclosed herein show that all of these drugs inhibit the action of CYP1B1 on its substrate EROD, and further experiments with paclitaxel and docetaxel confirm this result in cell assays. The CYP1B1 inhibitor and the anti-cancer agents may be formulated together in a composition or separately for simultaneous or sequential administration.

Preferably, the types of cancer treatable using the present invention are those characterised by the presence of CYP1B1 in tumour cells and more especially, the presence of CYP1B1 at an elevated level. Examples of

such types of cancer include breast cancer, kidney cancer, colorectal cancer, prostate cancer, liver cancer or ovarian cancer.

5   Accordingly, in a further aspect, the present invention provides a composition comprising a substance capable of inhibiting CYP1B1 protein and an anti-cancer drug, in combination with a physiologically acceptable carrier.

10 In a further aspect, the present invention provides a kit comprising:

    (a) in a first container, a substance capable of inhibiting CYP1B1 protein; and

    (b) in a second container, an anti-cancer drug;

15 wherein the substances are formulated with a physiologically acceptable carrier and are for simultaneous or sequential administration. The kit may also include instructions for administering the components of the kit.

20

In a further aspect, the present invention provides a method for treating cancer, the method comprising administering to a patient in need a combination of a substance capable of inhibiting CYP1B1 protein and an

25 anti-cancer drug.

In a further aspect, the present invention provides a method of screening for CYP1B1 inhibitors for use in combination with anti-cancer drugs, the method

30 comprising:

    (a) contacting a candidate substance with CYP1B1 protein under conditions where the candidate substance and CYP1B1 can interact;

    (b) measuring the activity of the CYP1B1 protein

35 and comparing the value obtained to standards; and

(c) selecting candidate compounds which have the effect of inhibiting CYP1B1.

In one embodiment, the method involves contacting CYP1B1 with a candidate compound and a substrate of CYP1B1 (e.g. EROD) and the effect of the candidate compound can be determined by monitoring the effect of the candidate compound in inhibiting the breakdown of the substrate by CYP1B1.

Optionally, the method may comprise the additional step of testing the candidate compounds, e.g. in combination with CYP1B1 and one or more anti-cancer drugs, to determine the effect the candidate inhibitor has in reducing the effect of CYP1B1 in inactivating or metabolising the anti-cancer drug. Examples of assays include the in vitro interaction assays described below and cell based assays measuring the effect of the inhibitors in reducing the loss of cytotoxicity of the anti-cancer drugs caused by CYP1B1 action.

In carrying out these methods, it may be convenient to screen a plurality of candidate compounds, e.g. as present in a library, at the same time, e.g. by contacting a mixture of different candidate compounds with the interacting peptides, and then in the event of a positive result resolving which member of the mixture is active. These techniques are used in high throughput screening (HTS) to increase the numbers of compounds, e.g. resulting from a combinatorial chemistry program or present in a library derived from a natural source material.

The precise format of the assays of the invention may be varied by those of skill in the art using routine skill

and knowledge. For example, interaction between
substances may be studied in vitro by labelling one with
a detectable label and bringing it into contact with the
other which has been immobilised on a solid support. The
amount of candidate substance or compound which may be
added to an assay of the invention will normally be
determined by trial and error depending upon the type of
compound used. Typically, from about 0.01 to 100 nM
concentrations of putative inhibitor compound may be
used, for example from 0.1 to 10 nM.

In a further aspect, the present invention provides a
method of determining the diagnosis, prognosis or
responsiveness to treatment of a patient having ovarian
cancer, the method comprising determining a presence or
amount of CYP1B1 protein in a sample from a patient
comprising ovarian cancer cells and correlating the
presence or amount to control values. Examples of assays
for use in this aspect of the invention are disclosed on
WO97/12246 and antibodies suitable for use in
immunoassays are disclosed in WO00/56773.

Embodiments of the present invention will now be
described by way of example and not limitation with
reference to the accompanying figures.

Brief Description of the Figures

Figure 1. Survival curves of cells treated with A,
docetaxel; B, paclitaxel; C, cyclophosphamide; D,
doxorubicin; E, 5-FU; F, carboplatin and G, cisplatin.
Parental V79MZ cells and those transfected with CYP1B1
(V79MZh1B1) were incubated with increasing concentrations
of the appropriate drug for 24h. Cell viability was then
determined by the MTT assay and the percentage of
surviving cells relative to the respective controls

(cells treated with solvent only), was calculated for each drug concentration. There was significantly different cytotoxicity for docetaxel (A) in CYP1B1 expressing and non-expressing cell lines and a lesser effect was observed with paclitaxel, perhaps because the action of CYP1B1 resulted in a product which retained some cytotoxicity. There was no cytotoxicity observed in cells exposed to cyclophosphamide (C). Each 96 well plate allowed eight concentrations of the appropriate drug per plate, with eight replicates (i.e. eight separate wells) per concentration. (i.e. 8 measurements for each concentration of drug per plate, There were three triplicate plates per experiment resulting in a total of 24 measurements of absorbance per concentration of drug). The standard deviation was less than 5% of the mean absorbance for all drugs used, and at each concentration of drug.

Figure 2 Survival curve of cells treated with docetaxel in the presence or absence of the cytochrome P450 inhibitor ANF. Parental V79MZ cells and those transfected with CYP1B1 (V79MZh1B1) were incubated with increasing concentrations of docetaxel for 24 hr. ANF was added at the following concentration 1, 10, or 100 μM to the transfected V79MZh1B1 cells. Cell viability was then determined by the MTT assay and the percentages of surviving cells, relative to the respective solvent controls, were calculated. ANF at a concentration of 100 μM totally abolished the differential cytotoxicity observed in the V79MZh1B1 transfected cells. ANF, itself, exhibited no cytotoxic effects on the parental (V79MZ) and CYP1B1 (V79MZh1B1) expressing cell line, at all of the concentrations used.

Figure 3. Survival of patients treated with docetaxel as

part of their anti-cancer drug regimen and classified
according to CYP1B1 status of their primary tumours. The
presence of strong/moderate CYP1B1 immunoreactivity
results in a poorer overall survival compared with weak
or absent CYP1B1 immunoreactivity.

Detailed Description

Pharmaceutical Compositions

The compounds described herein or their derivatives can
be formulated in pharmaceutical compositions, and
administered to patients in a variety of forms, in
particular to treat cancer, and more especially breast
cancer, colorectal cancer, prostate cancer, liver cancer
or ovarian cancer.

Pharmaceutical compositions for oral administration may
be in tablet, capsule, powder or liquid form. A tablet
may include a solid carrier such as gelatin or an
adjuvant or an inert diluent. Liquid pharmaceutical
compositions generally include a liquid carrier such as
water, petroleum, animal or vegetable oils, mineral oil
or synthetic oil. Physiological saline solution, or
glycols such as ethylene glycol, propylene glycol or
polyethylene glycol may be included. Such compositions
and preparations generally contain at least 0.1wt% of the
compound.

Parental administration includes administration by the
following routes: intravenous, cutaneous or subcutaneous,
nasal, intramuscular, intraocular, transepithelial,
intraperitoneal and topical (including dermal, ocular,
rectal, nasal, inhalation and aerosol), and rectal
systemic routes. For intravenous, cutaneous or
subcutaneous injection, or injection at the site of
affliction, the active ingredient will be in the form of

a parenterally acceptable aqueous solution which is pyrogen-free and has suitable pH, isotonicity and stability. Those of relevant skill in the art are well able to prepare suitable solutions using, for example, solutions of the compounds or a derivative thereof, e.g. in physiological saline, a dispersion prepared with glycerol, liquid polyethylene glycol or oils.

In addition to one or more of the compounds, optionally in combination with other active ingredient, the compositions can comprise one or more of a pharmaceutically acceptable excipient, carrier, buffer, stabiliser, isotonicizing agent, preservative or anti-oxidant or other materials well known to those skilled in the art. Such materials should be non-toxic and should not interfere with the efficacy of the active ingredient. The precise nature of the carrier or other material may depend on the route of administration, e.g. orally or parentally.

Liquid pharmaceutical compositions are typically formulated to have a pH between about 3.0 and 9.0, more preferably between about 4.5 and 8.5 and still more preferably between about 5.0 and 8.0. The pH of a composition can be maintained by the use of a buffer such as acetate, citrate, phosphate, succinate, Tris or histidine, typically employed in the range from about 1 mM to 50 mM. The pH of compositions can otherwise be adjusted by using physiologically acceptable acids or bases.

Preservatives are generally included in pharmaceutical compositions to retard microbial growth, extending the shelf life of the compositions and allowing multiple use packaging. Examples of preservatives include phenol,

meta-cresol, benzyl alcohol, para-hydroxybenzoic acid and
its esters, methyl paraben, propyl paraben, benzalconium
chloride and benzethonium chloride. Preservatives are
typically employed in the range of about 0.1 to 1.0 %
(w/v).

Preferably, the pharmaceutical compositions are given to
an individual in a "prophylactically effective amount" or
a "therapeutically effective amount" (as the case may be,
although prophylaxis may be considered therapy), this
being sufficient to show benefit to the individual.
Typically, this will be to cause a therapeutically useful
activity providing benefit to the individual. The actual
amount of the compounds administered, and rate and time-
course of administration, will depend on the nature and
severity of the condition being treated. Prescription of
treatment, e.g. decisions on dosage etc, is within the
responsibility of general practitioners and other medical
doctors, and typically takes account of the disorder to
be treated, the condition of the individual patient, the
site of delivery, the method of administration and other
factors known to practitioners. Examples of the
techniques and protocols mentioned above can be found in
Remington's Pharmaceutical Sciences, 16th edition, Osol,
A. (ed), 1980. By way of example, the compositions may
be administered to patients in dosages of between about
0.01 and 100mg of active compound per kg of body weight,
and more preferably between about 0.5 and 10mg/kg of body
weight.

Materials and Methods
Tissue
Samples of ovarian cancer (n=172) submitted to the
Department of Pathology, University of Aberdeen for
diagnosis, over a five year period (1993-1998), were used

in this study, with ethical approval from the Joint
Grampian Health Board and University of Aberdeen Research
Ethics Committee. Of the 172 cases of ovarian cancer,
167 cases were of the primary ovarian tumour, 43 of these
5   cases had samples of both primary and metastatic disease,
while 5 cases had samples of metastatic deposits with no
corresponding ovarian tumour for investigation, (i.e. a
total of 48 cases of metastatic disease and 167 cases of
primary ovarian tumour). In 49 cases contralateral
10   normal ovary was also submitted for histopathological
examination and available for study. All the tissue
samples had been fixed in 10% neutral buffered formalin
for 24 hours and then routinely processed to paraffin
wax. The diagnosis of ovarian cancer was performed with
15   hematoxylin and eosin stained sections using standard
histopathological criteria by a consultant
histopathologist with special interest in gynaecological
pathology. The tumours were graded according to criteria
described by FIGO. The median age of patients in this
20   study was 63 years with an age range from 30-89 years.
Information on therapeutic treatment and clinical outcome
was available for 170 patients. The following anti-
cancer drugs (cisplatin, carboplatic, cyclophosphamide,
paclitaxel, and docetaxel) were used to treat the
25   patients. Most patients received either cisplatin or
carboplatin. The other three drugs were usually given in
combination with a platinum based drug. Following
diagnosis, the disease status of patients was assessed at
regular intervals by two gynaecological oncologists with
30   the median overall survival of patients being 17 months.

Localisation of CYP1B1 in ovarian cancer
Immunohistochemical detection of CYP1B1 with a monoclonal
antibody to CYP1B1 was performed using a tyramine signal
35   amplification method as described previously [3]. Sites

of immunoreactivity were demonstrated colorimetrically with diaminobenzidine and hydrogen peroxide (Liquid DAB plus, Dako Ltd High Wycombe, Bucks, UK). Positive control tissue was sections of breast cancer, which had been previously shown to contain CYP1B1 by immunohistochemistry [3], the negative control used Tris buffered saline (TBS) in place of the primary monoclonal antibody. To establish the presence or absence of CYP1B1 and its distribution, intensity and cellular localisation, the sections were examined using bright field light microscopy by two independent observers. CYP1B1 immunoreactivity in the tumours was assessed as strong (3), moderate (2), weak (1), or negative (0). Tumours exhibiting CYP1B1 immunoreactivity in more than 5% of the cell were considered as positive.

Cell lines and cell culture
A Chinese hamster ovary fibroblast cell line (V79MZ) and a clone expressing human CYP1B1 (V79MZh1B1) [21], were grown at 37°C, 5%CO_2 and at 90% saturated humidity, in DMEM (Dulbecco's modified eagles medium) high glucose type supplemented with 1 mM sodium pyruvate, 10% fetal calf serum, 100 U penicillin/ml and 100 µg streptomycin/ml. Neither cell line expresses endogenous P450s, although cytochrome P450 reductase is present in both cell lines [22,23,24]. The parental and CYP1B1 expressing cell lines double in cell number every 10-12h [23] and were sub-cultured at 1:50 ratio (i.e. 1 ml of cells to 50 ml of fresh media) every 4-5 days. The cells were not allowed to reach confluency at any time, to ensure optimal cell physiological conditions and maximal cytochrome P450 activity [23]. Cells were routinely used at 3rd-5th passage for all experiments [21].

Immunoblotting of CYP1B1 protein in V79 total cellular

homogenate

Both cell lines (V79MZ and V79MZh1B1) were grown to 60-
80% confluence and a total cellular homogenate from each
prepared. Cellular protein was determined according to
the method of Bradford [25]. Samples of cellular
homogenates were then resolved by SDS-PAGE on a 10%
polyacrylamide gel using a Hoefer SE 600 (Amersham
Pharmacia Biotech) vertical gel electrophoresis system.
This was followed by transfer to nitrocellulose membrane
(Hybond-ECL, Amersham Pharmacia Biotech). Sites of
immunoreactivity were detected with a monoclonal antibody
to human CYP1B1 [3]. This antibody was raised against a
15 amino acid peptide corresponding to amino acid
residues 437-451 of the human CYP1B1 protein [3]. CYP1B1
immunoreactivity was visualised by an enhanced
chemiluminescence detection system (Amersham Pharmacia
Biotech). Microsomes prepared from human lymphoblastoid
cells which have been transfected to stably express human
CYP1B1 (Gentest) were used as the positive control [26].

Cell viability assay

Paclitaxel, cyclophosphamide, doxorubicin, 5-FU,
cisplatin, carboplatin and ANF were purchased from Sigma.
Docetaxel was obtained from Rhone-Poulenc Rorer. Optimal
growth conditions were established. The same conditions
were used for both cell lines; V79MZ and V79MZh1B1 cells
at 60-80% confluence were harvested and seeded at 0.5-1 x
10^3 cells per well in 96 well culture plates. Cells were
grown for 48 hours, media was then removed and each drug
added at increasing concentrations in the appropriate
solvent (0.1% ethanol for docetaxel, paclitaxel,
cyclophosphamide and carboplatin, 0.1% DMSO for
cisplatin, sterile water for 5-FU and doxorubicin).
Cells treated with solvent alone acted as a negative
control.

Enzyme inhibition studies were undertaken by co-administration of docetaxel and the known P450 CYP1 inhibitor ANF (Sigma) [27] at different concentration in the media. Stock solutions of ANF (1 mM, 10 mM and 100 mM) were dissolved in DMSO and added to the media to give final concentrations of 1 µM, 10 µM and 100 µM ANF (in each case the final concentration of DMSO in media was 0.1%). Following 24h exposure to each drug (with or without inhibitor), the media were removed and replaced with fresh media without drug. In this study, cells were then grown for three doubling times (36h) with the media changed at 24hr. Cell viability was then assessed using the MTT assay, which is comparable to using a clonogenic assay [23,28,29]. Media were removed from the wells and replaced with 200 µl of fresh media, followed by addition of 50 µl of MTT solution (50 mg/ml of MTT (Sigma) in sterile PBS), the cells were incubated at 37°C in a humidified atmosphere with 5%CO_2/95% air for 4h. The MTT containing media was then removed and 200µl of DMSO plus 25 µl of glycine buffer (0.1 M glycine/ 0.1 M NaCl pH10.5) added to the cells in each well. This procedure overcomes any effect that cell density or culture medium may have on the absorption spectrum [28]. The absorbance of the formazan produced by the viable cells was measured once for each well at 540nm on a Labsystems EMS microplate reader (Life Sciences International). To calculate the cell viability cells treated with solvent alone were assigned a value of 100% absorbance indicating zero cytotoxicity, i.e. 100% viability. The cytotoxic profile of each drug was evaluated in triplicate 96 well plates. Each plate included several controls (media only, cells only and cells treated with solvent alone). The 96 well plate format allowed eight concentrations of the appropriate drug per plate, with eight replicates (i.e. eight separate wells) per drug concentration, i.e.

. 8 measurements of absorbance for each concentration of drug per plate. There were three triplicate plates per experiment resulting in a total of 24 measurements of absorbance per concentration of drug. Plate reader

5   variability was found to be negligible.

P4501B1 has a role in metabolism of anticancer agents

Recent immunohistochemical analysis of P4501B1 protein in a variety of solid tumours identified tumour-specific

10 enzyme expression (Murray et al, 1998). This provides a mechanism of resistance to currently used anticancer agents, as well as, an approach for targeted prodrug therapy. 7-ethoxyresorufin deethylase activity (EROD) was used to define interactions with cytochrome 1B1

15 (CYP1B1). 7-Ethoxyresorufin is a known substrate for P4501B1. EROD activity was used to screen the binding affinity of anticancer agents and test compounds with CYP1B1. For this purpose, inhibition constants (Ki) have been calculated using *in vitro* kinetics from CYP1B1-

20 mediated EROD activity. EROD activity and assay has been described previously by various authors (Lubet et al., 1985, Arch. Biochem. Biophys. 238: pp 43-48; Gentest Corporation, 6 Henshaw St., Woburn, MA01801 USA, data sheet given with CYP1B1, catalogue number P220). 7-

25 Ethoxyresorufin is enzymatically deethylated to a product called resorufin. The appearance of resorufin was monitored by a fluorescence detector set at 550nm and 582nm (excitation and emission wavelengths). Various concentrations of 7-ethoxyresorufin (0.05, 0.1 and 0.5µM)

30 as well as various concentrations of anti-cancer agents and test (control) compounds (depending on the efficiency of the inhibition) were added to the incubation system. Incubations contained: phosphate buffer at pH7.4, 7-ethoxyresorufin, CYP1B1 Supersomes from Gentest Co

35 (catalogue number P220) and nicotine adenine dinucleotide

phosphate (NADP) as cofactor.

Biotransformation studies

Flutamide and paclitaxel (taxol) were tested as potential substrates of the CYP1B1. The results show that both drugs are biotransformed by CYP1B1, as metabolites were produced by CYP1B1 incubation but not control incubations.

Kidney tumour assays

The effect of a CYP1B1 inhibitor was confirmed in an assay using kidney tumour samples. A buffer and NADP regenerating system was allowed to equilibrate at 37°C and EROD (5 µM final concentration) was then added. This was followed by the addition of either P450 supersomes (control) or the appropriate tumour sample (1 mg). Supersomes were incubated for 15 minutes and tumour samples for 40 minutes. The CYP1B1 inhibitor α-naphthoflavone was added to some samples just prior to the addition of EROD and P450 sample.

Statistics

Statistical analysis was performed using both Statistics for Windows 95 and SPSS version 7.5 for Windows 95.

Results

Localisation of CYP1B1 in ovarian cancer

CYP1B1 immunoreactivity was identified in the majority (153/167; 92%) of primary ovarian cancer sections and was specifically localised to the cytoplasm of tumour cells. There was no detectable CYP1B1 expression in any of the normal ovarian tissue samples. In a high percentage of the ovarian cancers there was either strong (85/167; 50.9%) or moderate (39/167; 23.4%) immunoreactivity for

CYP1B1 (Table 4). In addition, the presence of CYP1B1
was observed in the majority (45/48; 95%) of metastatic
deposits with a high proportion showing moderate
(22/48;45.8%) to strong (18/48;37.5%) immunoreactivity

5   (Table 5). A similar level of CYP1B1 expression was
exhibited for the different histological subtypes in both
primary and metastatic tumour (Tables 4 and 5). In the
cases where both primary ovarian tumour and metastatic
deposits were available, a significant correlation for

10   CYP1B1 expression (p=0.05 Spearman correlation test) was
observed). The presence of moderate to strong CYP1B1 in
the tumours of the subset of 19 patients who had received
docetaxel as part of their anti-cancer drug regime had an
adverse effect on overall survival (Fig. 3). The

15   presence of CYP1B1 had no influence on the survival of
patients who had received other anti-cancer drug regimes.

In parallel with the *in vitro* studies of CYP1B1 on the
cytotoxic profile of the anti-cancer drugs, a

20   comprehensive investigation was conducted into the
presence of CYP1B1 in primary ovarian tumour and
metastatic deposits and the influence it has on the
overall survival of patients on different therapeutic
regimens. A monoclonal antibody to CYP1B1 was used to

25   demonstrate the localisation of CYP1B1 to ovarian tumour
cells and lack of expression in normal ovarian tissue.
The over-expression of CYP1B1 was observed in all
histological subtypes of epithelial ovarian cancer and at
high frequency (>85%). This finding is in agreement with

30   a previous study, which utilised a polyclonal antibody to
CYP1B1 to demonstrate the over-expression of CYP1B1 in a
small number (7/7) of ovarian serous adenocarcinomas (7).
A high frequency of CYP1B1 over-expression was also
observed in the majority of metastatic deposits in this

35   current study, and was highly correlated with paired

primary tumour. These findings also support the concept of CYP1B1 being a molecular target for the development of new treatment approaches to ovarian cancer.

During the time period in which patients on this study were diagnosed (1993-1998), considerable changes were observed in the chemotherapeutic regimen used for the treatment of ovarian cancer. Prior to 1994 the main treatment of choice was a cisplatin/cyclophosphamide based-regimen (19). Although a platinum agent is still regarded as essential for primary treatment, the less toxic agent carboplatin is now the platinum agent of choice. Following the introduction of paclitaxel to oncological practice, the principal first line therapy for ovarian cancer is currently carboplatin plus paclitaxel (19). Docetaxel, is currently under assessment including the "SCOTROC" trial (Scottish randomised trial on ovarian cancer comparing carboplatin plus paclitaxel versus carboplatin plus docetaxel), prior to possible licensing for treatment in ovarian cancer. These changes observed in primary treatment have resulted in a skewed grouping of patients on the various chemotherapeutic regimens in this study. However, the results of this investigation indicate that in the subset of patients treated with docetaxel either as a single agent or in combination with a platinum agent, the presence of CYP1B1 in the tumour resulted in a poorer overall survival, supporting the *in vitro* data. In summary this study provides evidence to support the concept that the presence of CYP1B1 in tumour cells may have an important role in drug resistance, especially to docetaxel.

Cytotoxic effects of treatment with anti-cancer drugs
A protein band of approximately 52kDa was identified in

the cellular homogenate from the V79MZh1B1 cell line,
corresponding to the expected molecular size observed
with lymphoblastoid cells which also express human CYP1B1
[3]. No immunoreactive band was observed in the parental

5   V79MZ cell line. This confirms the presence of CYP1B1
immunoreactivity in the CYP1B1 transfected cells and an
absence of CYP1B1 in the parental non-transfected cells.

The influence of CYP1B1 on cytotoxicity was evaluated for

10   seven of the fourteen anti-cancer agents investigated.
The range of concentrations for each drug used in this
study was based on previous experiments with other P450s
[23]. A significantly greater (at least four-fold)
decreased sensitivity to docetaxel was observed in cells

15   expressing CYP1B1 compared with non-CYP1B1 expressing
cells (Fig. 1A. and Table 1). The cytochrome P450 CYP1
inhibitor ANF was used at serial concentrations (1, 10
and 100 µM) to determine if the differential cytotoxicity
demonstrated in the cells expressing CYP1B1 was due to

20   metabolism by CYP1B1 (Fig. 2). Neither cell line
(parental or CYP1B1 expressing) showed cytotoxicity on
exposure to any of the concentrations of ANF. In
contrast to the decreased sensitivity observed with
docetaxel, no significant difference in cytotoxicity was

25   observed between CYP1B1 expressing and non-expressing
cells after exposure to paclitaxel (Fig. 1B. and Table
1). No cytotoxicity was observed in either cell line
after exposure to cyclophosphamide (Fig. 1C and Table 1).
In addition, no significant difference in cytotoxicity

30   was observed between V79MZ and V79MZh1B1 cells after
exposure to doxorubicin, 5-FU, carboplatin or cisplatin
(Fig. 1D-1G; Table 1).

P4501B1 has a role in metabolism of anti-cancer agents

35   The metabolism of anti-cancer drugs by CYP1B1 was studied

using an inhibition of EROD assay. Control incubations
(with similar amounts of drug vehicle) have shown that
inhibition of EROD activity was due to the anti-cancer
and test compounds mentioned in the attached results. Ki

5   values were determined by the Dixon's replots: see Table
2. The low Ki values show that CYP1B1 has a strong
binding affinity for many anti-cancer agents and could
biotransform these drugs.

10 Many anti-cancer agents showed strong binding affinity
(Ki values) on cytochrome 1B1. Moreover, the two anti-
cancer agents tested in biotransformation studies,
paclitaxel and flutamide, are biotransformed by CYP1B1.
Therefore, CYP1B1, which is over-expressed in tumour

15 cells, could have important consequences in the
development of new drugs of new therapies and in the
prediction of therapy outcome.

Inhibition of CYP1B1

20 As initial screening for the inhibition of CYP1B1
activity, resorufin production was measured with 1 µM
ethoxyresorufin in the presence of 0 µM (as control) or
100 µM anticancer agent. In these conditions,
vinblastine, vincristine, 5-fluorouracil, etoposide, and

25 cyclophosphamide did not inhibit CYP1B1 activity. In
contrast, flutamide, paclitaxel, mitoxantrone, docetaxel,
doxorubicin, daunomycin, and tamoxifen inhibited CYP1B1
activity by decreasing the production of resorufin by 53
to 99%. Further inhibition studies performed with three

30 concentrations of ethoxyresorufin and six concentrations
of drugs identified flutamide, mitoxantrone, docetaxel,
and paclitaxel as competitive inhibitors with K_i values of
1.0, 11.6, 28.0, and 7.85 µM respectively (Table 2).
Noncompetitive or mixed inhibition was observed for

35 daunomycin, doxorubicin, and tamoxifen, and K_i values were

2.1, 2.6, and 5.0 µM, respectively (Table 2).

Similarly, known CYP inhibitors and putative CYP1B1
substrates were also initially screened at 100 µM and
agents with an apparent interaction were further
characterised as described above. Erythromycin and
cyclosporine did not inhibit CYP1B1 activity (10%; Table
2). In contrast, testosterone and estradiol were
competitive inhibitors of EROD (K_i=1.9 and 411.8 µM
respectively). Potent non-competitive inhibition by
ketoconazole (K_i = 0.3 µM) and α-naphthoflavone (K_i = 2.8
µM) was observed.

Flutamide Metabolism

Flutamide was a potent competitive inhibitor of CYP1B1,
suggesting that it is a putative substrate. In vitro
incubations of flutamide were performed with human liver
microsomes or various cDNA-expressed human CYPs. Two
flutamide metabolites were produced in the presence of a
NADP-regenerating system. One metabolite was observed
after incubation with CYP1B1, CYP1A1, or CYP1A2. This
metabolite has been identified as 2-hydroxyflutamide,
following metabolic studies of CYP1B1 and CYP1A1
activities. As previously reported (Shet et al, 1997),
the production of the other metabolite is via CYP3A4
activity.

The 2-hydroxylation of flutamide was produced by
microsomes containing human CYP1B1, CYP1A1, and CYP1A2.
Production of 2-hydroxyflutamide by CYP1B1 was described
by Michaelis-Menten kinetics, and K_m and V_{max} values were
calculated by Eadie-Hofstee plots. Flutamide was a
competitive inhibitor of CYP1B1, CYP1A1, and CYP1A2
activities, with K_i values ranging from 1.0 to 10.3 µM.
For CYP1B1 and CYP1A2, similar K_m and K_i values were

obtained. In contrast, K_m and K_1 values for CYP1A1 are 5 and 10 times higher than CYP1B1, respectively. V_{max} values for 2-hydroxylation of flutamide are different for all three enzymes, likely reflecting differences in cytochrome c reductase activity in the microsome preparation used. In this study, the cytochrome c reductase activity was 310, 1600, and 2330 mnol/min x mg of proteins for CYP1B1, CYP1A1, and CYP1A2, respectively.

Kidney tumour cell assay

These results of this assay confirms that an inhibitor of CYP1B1 can inhibit the protein when it is present in kidney tumour samples, providing confirmation that the inhibitors can be employed with anti-cancer agents. Three separate tumour samples were used and the CYP1B1 activity in metabolising EROD was measured in the presence and absence of the inhibitor α-naphthoflavone.

Sample 1: CYP1B1 activity   252 fmol/min/mg protein
                plus 10nM ANF   145 fmol/min/mg protein

Sample 2: CYP1B1 activity   993 fmol/min/mg protein
                plus 10nM ANF   470 fmol/min/mg protein

Sample 3: CYP1B1 activity   880 fmol/min/mg protein
                plus 10nM ANF 570 fmol/min/mg protein

Benzpyrene assay

This assay can be used to measure CYCP1B1 activity in tumour samples, and in particular in frozen sections of tumour. In the assay, fresh, frozen sections of tumour were incubated with benzpyrene (10-100μM and NADPH for 1 hour at 37°C and then mounted in alkaline glycerol jelly. The presence of 1B1 was visualised by fluorescence microscopy using excitation at 400-450nm and detecting

emission at 520nm through a dichroic mirror having a
510nm frequency cut off. Yellow-green fluorescence is
observed in tumour cells.

5   **Discussion**

In this study a Chinese hamster cell line which stably
expresses human CYP1B1 [21] was used as a bioassay to
assess the effect of CYP1B1 on the cytotoxicity of a
range of anti-cancer drugs. Although several of the

10   drugs used in this study are clinically relevant in the
treatment of ovarian cancer, i.e. cisplatin, carboplatin
and 5-FU [30,31], they have no known interactions with
cytochrome P450 enzymes. However, these drugs were used
to provide controls to assess the validity of the

15   cytotoxicity assay. The pro-drug cyclophosphamide
provided an appropriate negative control (i.e. non-
cytotoxic to either parental or CYP1B1 expressing cells)
as it requires activation by other cytochrome P450
enzymes (CYP2B6 and CYP3A4) before becoming cytotoxic,

20   but we have shown previously that it does not interact
with CYP1B1 [32]. Doxorubicin is known to be metabolised
to more cytotoxic compounds by the action of other
cytochrome P450 enzymes (CYP3A) [20], whereas the taxanes
paclitaxel and docetaxel are both metabolised to

25   pharmacologically less active metabolites by cytochrome
P450 enzymes; (CYP2C8 and CYP3A4 for paclitaxel and
CYP3A4 for docetaxel) [12,13,14,15,33]. Previous studies
have demonstrated that V79MZ cells when stably
transfected with other cytochrome P450 enzymes have

30   exhibited appropriate cytotoxicity when exposed to
individual anti-cancer drugs establishing this cell line
as an appropriate model for investigating anti-cancer
drug cytotoxicity [23].

35   It is widely known that both primary cultures and human

tumour cell lines rapidly lose the ability to
constitutively express cytochrome P450 enzymes in
culture.   Indeed, we have shown that the MCF-7 human
breast carcinoma and PEO4 ovarian adenocarcinoma derived
cell lines do not express CYP1B1 (unpublished
observations) even though we have shown over-expression
of CYP1B1 in both breast and ovarian tumours [1].   The
lack of constitutive expression of cytochrome P450s in
tumour cell lines was overcome by the use of a stably
transfected cell line expressing CYP1B1.   CYP1B1 activity
in these cells was previously shown to be approximately
10 pmol/min/mg of protein by the EROD assay [21], and is
likely to be comparable with that observed in human
tumours.

These results show that docetaxel is metabolised by
expressed human CYP1B1 [32]. Docetaxel is a semi-
synthetic taxane derived from the European yew, and is
currently under investigation, for use as first line
treatment of ovarian cancer [12-13,34].   The key finding
of the current study was the significant differential
cytotoxicity observed on exposure to docetaxel, the
cytotoxicity observed in CYP1B1 expressing cells was
four-fold less than that observed in non-CYP1B1
expressing cells.   In addition co-treatment of these
cells with the known CYP1 P450 inhibitor ANF [27]
resulted in the complete reversal of differential
cytotoxicity observed in these cells, i.e. the effect is
attributed to metabolism of docetaxel by CYP1B1.   The
resistance to the cytotoxic effects of docetaxel in those
cells expressing CYP1B1 may have important clinical
implications.

Inhibition of CYP1B1 in tumours may offer a specific
mechanism for overcoming the resistance to docetaxel and

other drugs. Development of a specific inhibitor to CYP1B1 is clinically important as ANF also inhibits CYP1A1 and CYP1A2 [27]. Since CYP1B1 is over-expressed in tumour but not in normal tissue, increasing the tumour sensitivity to anti-cancer drugs by CYP1B1 inhibition would not be expected to have an effect on normal tissues.

In summary, this study provides evidence for the concept that the presence of CYP1B1 in tumour cells may have an important role in drug resistance.

Table 1.

[a]IC50 values for V79MZ and V79MZh1B1 cell lines treated with several anti-cancer drugs

Drug	V79MZ (control vector)	V79MZhB1	p[*]-value
Docetaxel	22nM	100nM	0.03
Paclitaxel	35nM	60nM	NS
Cyclophosphamide	NC	NC	ND
Doxorubicin	80nM	90nM	0.79
5-Fluorouracil	70μM	80μM	NS
Carboplatin	80μM	100μM	NS
Cisplatin	4.4μM	6μM	NS

Statistical comparison of dose response curves (IC50s) using Mann-Whitney U test. [a]IC50= Drug concentration at which 50% of the cells are viable. The data represent the means of 24 determinations per drug concentration. NC = No cytotoxicity (no cytotoxicity observed with cyclophosphamide), ND = Not determined, NS = not statistically significant for paclitaxel, 5FU, cisplatin or carboplatin. The statistical software package we used did not provide p values in many cases when the result was non significant.

Table 2.

Cytochrome 1B1: Ki values

Ki values calculated from Dixon re-plots of EROD activity mediated by cDNA expressed cytochrome 1B1

Anticancer Agents	Inhibition Type	Ki Values	
Flutamide	competitive	mean	0.99µM
		sd	0.07µM
Tamoxifen	non-competitive	mean	5.02µM
		sd	0.08µM
Mitoxantrone	competitive	mean	11.63µM
		sd	0.03µM
Paclitaxel	competitive	mean	7.85µM
		sd	0.91µM
Docetaxel	competitive	mean	28.03µM
		sd	9.78µM
Doxorubicin	mixed	mean	2.58µM
		sd	0.18µM
Daunomycin	mixed	mean	2.12µM
		sd	0.11µM

Test Compounds	Inhibition Type	Ki Values	
α-naphthoflavone	non-competitive	mean	2.80nM
		sd	0.53nM
Testosterone	competitive	mean	411.83µM
		sd	40.38µM
Estradiol	competitive	mean	1.85µM
		sd	0.06µM
Ketoconazole	non-competitive	mean	0.27µM
		sd	0.01µM

Table 3.

Clinicopathological characteristics of patients with ovarian carcinoma

Age of Patients	
Median (Range)	63 years (30-89 years)
Stage	
1	44/172 (25%)
2	15/172 (9%)
3	103/172 (60%)
4	10/172 (6%)
Histology	
Serous cystadenocarcinoma	102/172 (59%)
Endometrioid carcinoma	35/172 (21%)
Mucinous cystadenocarcinoma	24/172 (14%)
Clear cell adenocarcinoma	7/172 (4%)
Malignant mixed Mullerian tumour	4/172 (2%)

Table 4.

The number of cases showing CYP1B1 expression in the different histological types of primary ovarian carcinoma

CYP1B1 immunoreactivity					
Diagnosis	Negative	Weak	Moderate	Strong	Total
Serous cystadenocarcinoma	8	15	22	54	99
Endometrioid carcinoma	2	7	13	13	35
Mucinous cystadenocarcinoma	3	4	4	12	23
Clear cell adenocarcinoma	1	3	0	3	7
Malignant mixed Mullerian tumour	0	0	0	3	3
Total	14	29	39	85	167

Table 5.

The number of cases of CYP1B1 expression in the different histological types of metastatic epithelial ovarian carcinoma

CYP1B1 immunoreactivity					
Diagnosis	Negative	Weak	Moderate	Strong	Total
Serous cystadenocarcinoma	2	4	20	13	39
Endometrioid carcinoma	0	1	0	4	5
Mucinous cystadenocarcinoma	0	0	2	0	2
Malignant mixed Mullerian tumour	1	0	0	1	2
Total	3	5	22	18	48

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incorporated by reference.

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Claims:

1.   Use of a substance capable of inhibiting CYP1B1 protein and an anti-cancer drug for the preparation of a medicament for the treatment of cancer.

2.   The use of claim 1, wherein the substance capable of inhibiting CYP1B1 protein inhibits an activity of CYP1B1 enzyme in metabolising or inactivating the anti-cancer drug or a pro-drug form thereof, or a metabolic product of the anti-cancer drug or pro-drug.

3.   The use of claim 2, wherein CYP1B1 causes a loss of cytotoxicity of the anti-cancer drug, or in the case of a pro-drug, the capacity to be converted to active drug.

4.   The use of any one of the preceding claims, wherein the substance capable of inhibiting CYP1B1 protein is a flavonoid, a flavone or ethenyl pyrene.

5.   The use of claim 4, wherein the substance is α-naphthoflavone.

6.   The use of any one of the preceding claims, wherein the anti-cancer drug is a docetaxel, paclitaxel, flutamide, tamoxifen, mitoxantrone, doxorubicin or daunomycin.

7.   The use of any one of the preceding claims, wherein the medicament is used for the treatment of breast cancer, kidney cancer, colorectal cancer, prostate cancer, liver cancer or ovarian cancer.

8.   The use of any one of the preceding claims, wherein the substance capable of inhibiting CYP1B1 protein and the anti-cancer agent are formulated together in a

composition.

9.   The use of any one of claims 1 to 7, wherein the
substance capable of inhibiting CYP1B1 protein and the
anti-cancer agent are formulated for sequential
administration.

10.   A composition comprising a substance capable of
inhibiting CYP1B1 protein and an anti-cancer drug, in
combination with a physiologically acceptable carrier.

11.   The composition of claim 10, wherein the substance
capable of inhibiting CYP1B1 protein inhibits an activity
of CYP1B1 enzyme in metabolising or inactivating the
anti-cancer drug or a pro-drug form thereof, or a
metabolic product of the anti-cancer drug or pro-drug.

12.   The composition of claim 11, wherein CYP1B1 causes a
loss of cytotoxicity of the anti-cancer drug, or in the
case of a pro-drug, the capacity to be converted to
active drug.

13.   The composition of any one of claims 10 to 12,
wherein the substance capable of inhibiting is a
flavonoid, a flavone or ethenyl pyrene.

14.   The composition of claim 13, wherein the substance
is α-naphthoflavone.

15.   The composition of any one of claims 10 to 14,
wherein the anti-cancer drug is docetaxel, paclitaxel,
flutamide, tamoxifen, mitoxantrone, doxorubicin or
daunomycin.

16.   A kit comprising:

(a)   in a first container, a substance capable of
inhibiting CYP1B1 protein; and,

(b)   in a second container, an anti-cancer drug,

wherein the substances are formulated with a

5   physiologically acceptable carrier and are for

simultaneous or sequential administration.

17.   A method for treating cancer, the method comprising

administering to a patient in need a combination of a

10   substance capable of inhibiting CYP1B1 protein and an

anti-cancer drug.

18.   A method of screening for GYP1B1 inhibitors for use

in combination with anti-cancer drugs, the method

15   comprising:

(a)   contacting a candidate substance with CYP1B1

protein under conditions where the candidate substance

and CYP1B1 can interact;

(b)   measuring the activity of the CYP1B1 protein

20   and comparing the value obtained to standards; and

(c)   selecting candidate compounds which have the

effect of inhibiting CYP1B1.

19.   The method of claim 18, further comprising the step

25   of testing the candidate compounds in combination with

CYP1B1 and one or more anti-cancer drugs to determine

whether the effect the candidate inhibitor has in

reducing the effect of CYP1B1 in inactivating or

metabolising the anti-cancer drug.

30

20.   The method of claim 19, wherein the testing step

employs an in vitro interaction assays or a cell based

assays measuring the effect of the inhibitors in reducing

the loss of cytotoxicity of the anti-cancer drugs caused

35   by CYP1B1 action.

21. A method of determining the diagnosis, prognosis or responsiveness to treatment of a patient having ovarian cancer, the method comprising determining a presence or amount of CYP1B1 protein in a sample from a patient comprising ovarian cancer cells and correlating the presence or amount to control values.
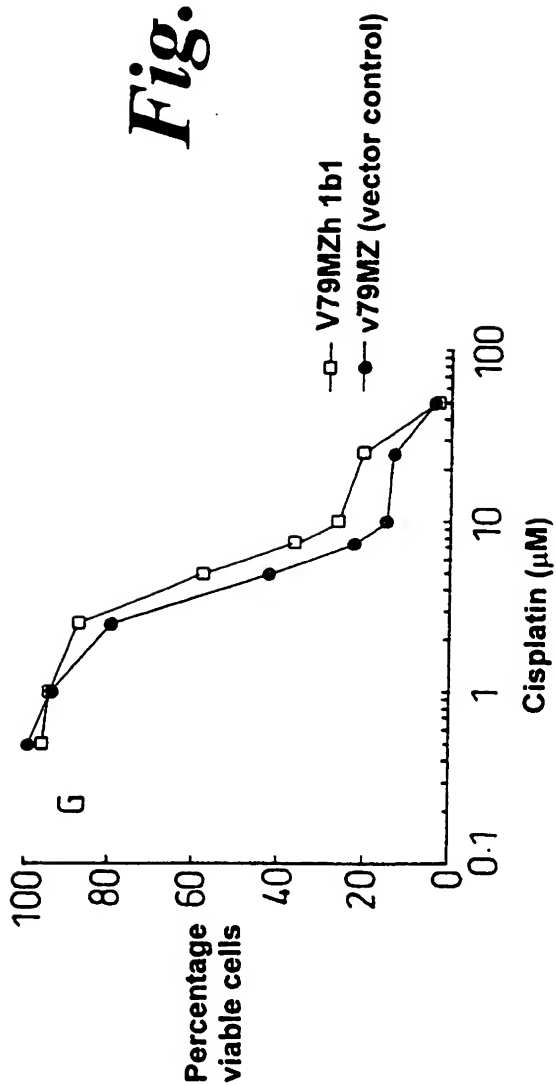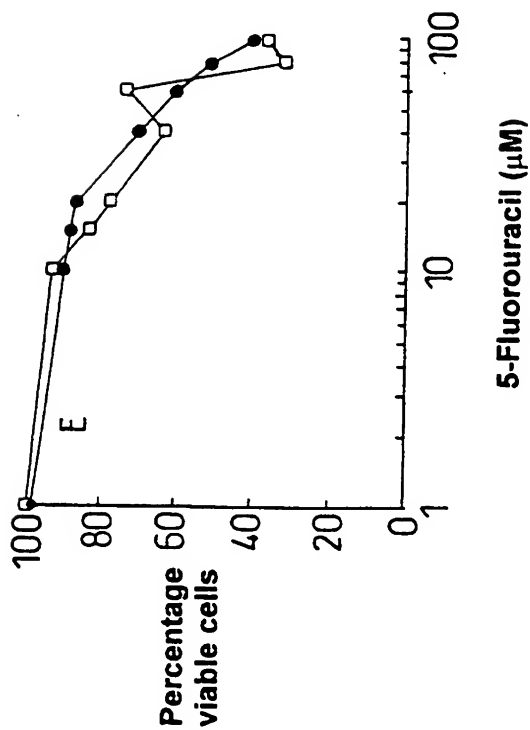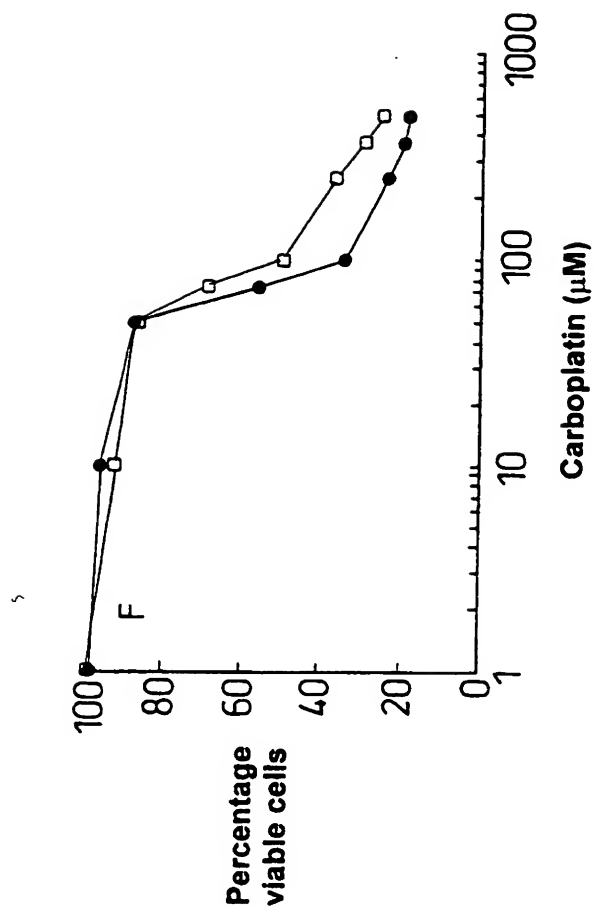
Fig. 1 (Part 1 of 2)

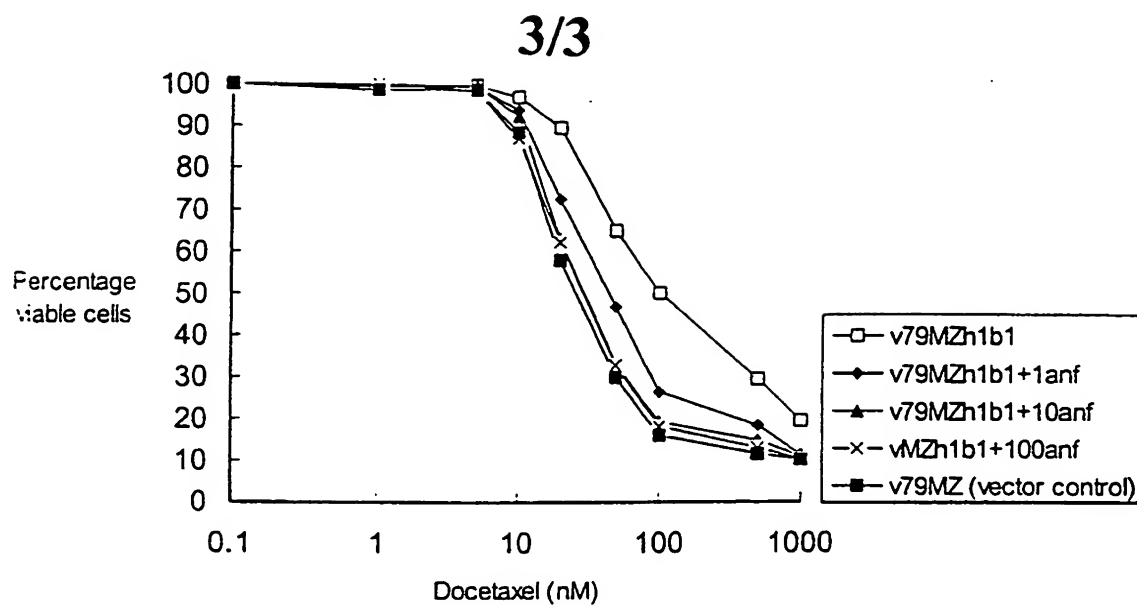*Fig. 1 (Part 2 of 2)*

3/3
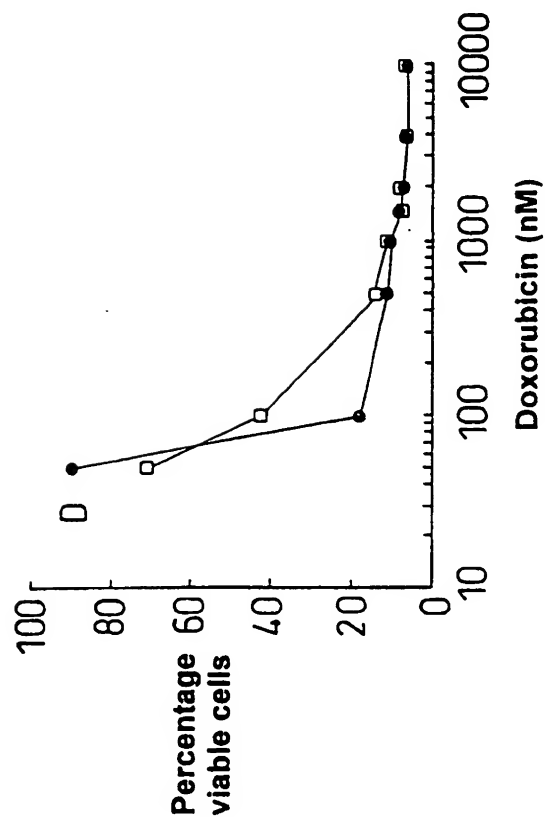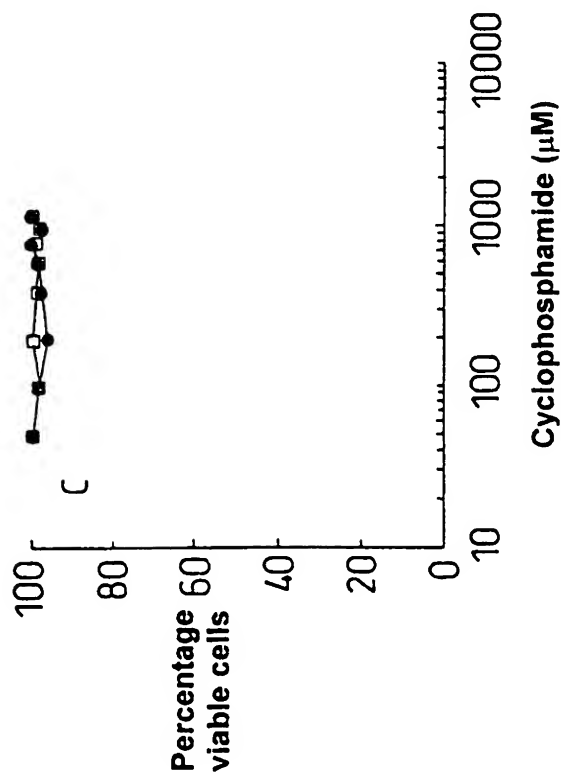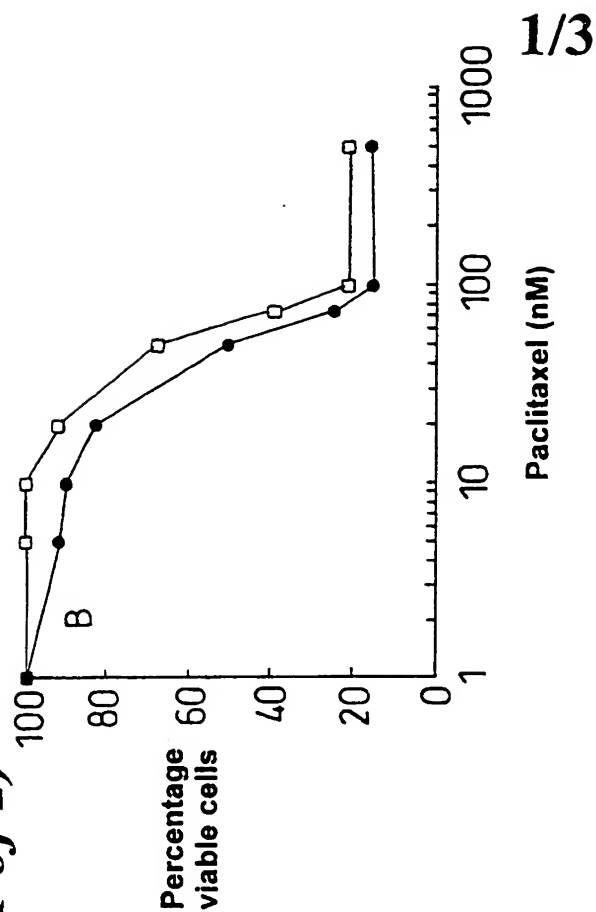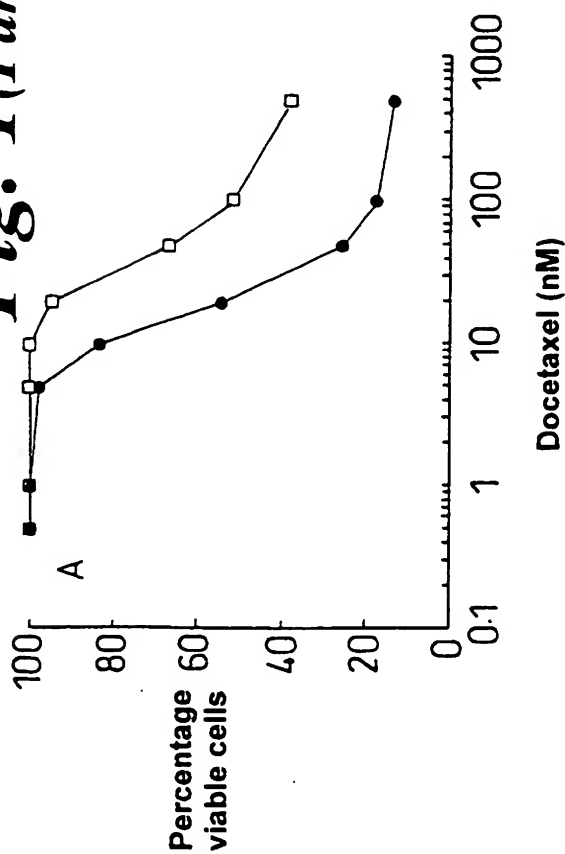
Fig. 2

Fig. 3

INTERNATIONAL SEARCH REPORT

A. CLASSIFICATION OF SUBJECT MATTER

IPC 7   A61K31/35   A61K31/135   A61K31/335   A61K31/165   A61K31/70
       A61P35/00   G01N33/574

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC 7   A61K   A61P

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

EPO-Internal, WPI Data, PAJ, MEDLINE, CHEM ABS Data, CANCERLIT, EMBASE, BIOSIS

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 99 15167 A (KGK SYNERGIZE INC ;KUROWSKA ELZBIETA MARIA (CA); GUTHRIE NAJLA (CA) 1 April 1999 (1999-04-01) * see pages 3,6-7,13, claims 1,3,7 and 11 *	1-4,6-8, 10-13, 15,17
X	TANG M. Y. ET AL.: "Development of an antipeptide antibody that binds to the C-terminal region of CYP1B1." DRUG METABOLISM AND DISPOSITION., vol. 27, no. 2, 1999, pages 274-280, XP000914872 * see abstract, fig.7 and page 277 left col.*	18

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[X] Further documents are listed in the continuation of box C.   [X] Patent family members are listed in annex.

° Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

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"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
10 May 2001	13/06/2001

Name and mailing address of the ISA European Patent Office, P.B. 5818 Patentlaan 2 NL – 2280 HV Rijswijk Tel. (+31–70) 340–2040, Tx. 31 651 epo nl, Fax: (+31–70) 340–3016	Authorized officer Merckling, V

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page 1 of 2

INTERNATIONAL SEARCH REPORT

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication,where appropriate, of the relevant passages	Relevant to claim No.
X	WO 97 12246 A (BURKE MICHAEL DANNY ;MELVIN WILLIAM THOMAS (GB); MURRAY GRAEME IAN) 3 April 1997 (1997-04-03) * see claims 1, 20 and 25 * ---	21
X,P	WO 00 56773 A (MURRAY GRAEME IAN ;UNIV ABERDEEN (GB); MELVIN WILLIAM (GB)) 28 September 2000 (2000-09-28) * see claims 1, 21 and 24 * ---	21
Y	KUPFER D. ET AL.: "Induction of tamoxifen-4-hydroxylation by TCDD, beta-naphthoflavone and phenobarbital in avian liver : identification of P450 TCDD(AA) as catalyst of 4-hydroxylation induced by TCDD and beta-naphthoflavone. " CANCER RESEARCH, vol. 54, no. 12, 1994, pages 3140-3144, XP000995373 * see abstract and page 3142 left col. * ---	1-21
Y	SHIMADA T. ET AL.: "Inhibition of human cytochrome P450-catalyzed oxidation of xenobiotics and procarcinogens by synthetic organoselenium compounds." CANCER RESEARCH, vol. 57, no. 21, 1997, pages 4757-4764, XP002167136 * see abstract and page 4761 right col. * -----	1-21

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INTERNATIONAL SEARCH REPORT

Information on patent family members

Patent document cited in search report		Publication date	Patent family member(s)		Publication date
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			EP	1049464 A	08-11-2000
WO 9712246	A	03-04-1997	AU	7090396 A	17-04-1997
			EP	0856157 A	05-08-1998
			JP	11512818 T	02-11-1999
WO 0056773	A	28-09-2000	AU	3308100 A	09-10-2000